CRYSTALLIZATION AND STRUCTURE DETERMINATION OF GLYCOLSYLATED HUMAN BETA SECRETASE, AN ENZYME IMPLICATED IN ALZHEIMER'S DISEASE

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FIELD OF THE INVENTION

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This invention relates to the crystallization and structure determination of beta secretase from human (*Homo sapiens*), particularly as expressed in Chinese hamster ovary (CHO) or HEK293 cells in its fully glycosylated form.

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BACKGROUND

Alzheimer's disease (AD) causes progressive dementia with consequent formation of amyloid plaques, neurofibrillary tangles, gliosis and neuronal loss. The disease occurs in both genetic and sporadic forms whose clinical course and pathological features are quite similar. Three genes have been

20 discovered to date which, when mutated, cause an autosomal dominant form of Alzheimer's disease. These encode the amyloid protein precursor (APP) and two related proteins, presenilin-1 (PS1) and presenilin-2 (PS2), which, as their names suggest, are structurally and functionally related. Mutations in any of the three proteins have been observed to enhance proteolytic processing of APP via an

25 intracellular pathway that produces amyloid beta peptide (Aβ peptide ,or sometimes here as Abeta), a 40-42 amino acid long peptide that is the primary component of amyloid plaque in AD.

Dysregulation of intracellular pathways for proteolytic processing

30 may be central to the pathophysiology of AD. In the case of plaque formation, mutations in APP, PS1 or PS2 consistently alter the proteolytic processing of APP so as to enhance formation of Aβ 1-42, a form of the Aβ peptide which seems to be

particularly amyloidogenic, and thus very important in AD. Different forms of APP range in size from 695-770 amino acids, localize to the cell surface, and have a single C-terminal transmembrane domain. Examples of specific isotypes of APP

5 which are currently known to exist in humans are the 695-amino acid polypeptide described by Kang *et. al.* (1987), *Nature 325*: 733-736 which is designated as the ⬛normal⬛ APP; the 751 amino acid polypeptide described by Ponte *et al.* (1988), *Nature 331*: 525-527 (1988) and Tanzi *et al.* (1988), *Nature 331*: 528-530; and the 770 amino acid polypeptide described by Kitaguchi *et. al.* (1988), *Nature 331*:

10 530-532. The Abeta peptide is derived from a region of APP adjacent to and containing a portion of the transmembrane domain. Normally, processing of APP at the α-secretase site cleaves the midregion of the $A\beta$ sequence adjacent to the membrane and releases the soluble, extracellular domain of APP from the cell surface. This α-secretase APP processing creates soluble APP- α, which is normal

15 and not thought to contribute to AD. Pathological processing of APP at the β- and γ-secretase sites, which are located N-terminal and C-terminal to the α-secretase site, respectively, produces a very different result than processing at the α site. Sequential processing at the β- and γ-secretase sites releases the $A\beta$ peptide, a peptide possibly very important in AD pathogenesis. Processing at the β- and

20 γ-secretase sites can occur in both the endoplasmic reticulum (in neurons) and in the endosomal/lysosomal pathway after reinternalization of cell surface APP (in all cells). Despite intense efforts, for 10 years or more, to identify the enzymes responsible for processing APP at the β and γ sites, to produce the $A\beta$ peptide, those proteases remained unknown until recently.

25   The identification and characterization of the β secretase enzyme, termed Aspartyl Protease 2 (Asp2) has established (Vassar *et. al.* (1999), *Science 286*:735-741; Yan *et. al.* (1999), *Nature 402*:533-537; Sinha *et. al.* (1999), *Nature 402*:537-540; Hussain *et. al.* (1999), *Mol. Cell Neurosci. 14*:419-427; Lin *et. al.* (2000), *Proc. Natl. Sci. USA 97*:1456-1460).

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In addition, the X-ray crystal structure of human beta secretase in complex with a peptide inhibitor was solved and published Hong *et al.* (2000), *Science 290*: 150-153; from protein expressed in *E. coli* that contained no covalent

5   sugar (glycosylation) at any of the four putative glycosylation sites within the enzyme.

SUMMARY OF THE INVENTION

This invention describes the crystallization and structure

10   determination of human beta secretase expressed in Chinese hamster ovary (CHO) or HEK 293 cells that contains covalent sugar at each of the four glycosylation sites.

In one aspect, the present invention provides a method for crystallizing a human beta secretase molecule or molecular complex. The method involves crystallizing a human beta secretase molecule or molecular complex by

15   preparing purified human beta secretase in the presence of a peptide mimetic inhibitor and crystallizing human beta secretase from a solution having a pH of about 3.5 to about 5.5.

In another aspect, the present invention provides crystalline forms of a human beta secretase molecule. In one embodiment, a crystal of human beta

20   secretase is provided having the trigonal space group symmetry $P32_21$ with unit cell dimensions of a, b, and c, wherein a is about 92 Å to about 132 Å, b is about 92 Å to about 132 Å, and c is about 90 Å to about 130 Å; and $\alpha=\beta=90°$, and $\gamma=120°$. Preferably, having unit cell dimensions of a=112.0 Å, b=112 Å, c=110 Å, $\alpha=\beta=90°$, $\gamma=120°$.

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DEFINITIONS

Two crystallographic data sets (with structure factors F) are considered isomorphous if, after scaling,

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$$\frac{\Delta F}{F} = \frac{\sum_i |F_1 - F_2|}{\sum F_1}$$

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is less than about 35% for the reflections between 8 Å and 4 Å.

BRIEF DESCRIPTION OF THE FIGURES

15   Figure 1 is an illustration of the chemical structures of inhibitors used in co-crystallization experiments. Figure 1(A) is the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine). Figure 1(B) is another inhibitor.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

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Crystalline Form(s) and Method of Making

The three-dimensional structure of human beta secretase was solved using high resolution x-ray crystallography to 3.2 Å resolution. Accordingly, the

25   invention includes a human beta secretase crystal and/or a crystal with human beta secretase co-crystallized with a ligand, such as an inhibitor. Preferably, the crystal has trigonal space group symmetry $P32_21$. More preferably, the crystal comprises hexagonal shaped unit cells, each unit cell having dimensions a=112.0 ± 20 Å, b=112 ± 20 Å, c=110 ± 20 Å, $\alpha=\beta=90°$, $\gamma=120$. The crystallized enzyme is a

30   monomer with a single monomer in the asymmetric unit.

According to the present invention, human beta secretase can be

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isolated from a variety of cell lines, such as the mammalian cell line CHO-K1, insect cell line for example.

5   In a preferred embodiment, molecular complexes of purified human beta secretase at a concentration of about 1 mg/ml to about 80 mg/ml may be crystallized in the presence of an inhibitor at a concentration from about 0.1 to about 10mM, for example, by using a streak seeding procedure from a solution including about 5 wt-% to about 50 wt-% PEG or PEG-MME (PEG monomethyl ether) or PEG-DME (PEG dimethyl ether) (preferably having a number average molecular

10   weight between about 200 and about 20,000), preferably, a salt (more preferably about 0.01 M to about 0.5 M salt), and about 0 wt-% to about 20 wt-% organic solvent (such as DMSO), wherein the solution is buffered to a pH of about 3.5 to about 5.5 (preferably, a pH of about 4.2 to about 4.5). Examplary salts include sodium chloride, ammonium sulfate, magnesium sulfate, lithium sulfate, or

15   combinations thereof. Use of a buffer having a pK_a of about 3 to about 6 is preferred. A "molecular complex" means a protein in covalent or non-covalent association with a chemical entity. A buffer having a pK_a of between about 3 and 6 is preferred for use in the crystallization method. A particularly preferred buffer is about 10mM to about 200mM sodium acetate. Variation in buffer and buffer pH as

20   well as other additives such as PEG or PEG-MME (PEG monomethyl ether) or PEG-DME (PEG dimethyl ether) is apparent to those skilled in the art and may result in similar crystals.

      The invention further includes an human beta secretase crystal that is isomorphous with an human beta secretase crystal characterized by a unit cell

25   having dimensions of a, b, and c; wherein a is about 92 Å to about 132 Å, b is about 92 Å to about 132 Å, and c is about 90 Å to about 130 Å; and $\alpha=\beta=90°$, and $\gamma=120°$.

X-ray Crystallographic Analysis

      Each of the constituent amino acids of human beta secretase is

defined by a set of structure coordinates. The term "structure coordinates" refers to Cartesian coordinates derived from mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of x-rays by the atoms (scattering

5   centers) of an human beta secretase complex in crystal form. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are then used to establish the positions of the individual atoms of the human beta secretase protein or protein/ligand complex.

Slight variations in structure coordinates can be generated by

10   mathematically manipulating the human beta secretase or human beta secretase/ligand structure coordinates. For example, structure coordinates could be manipulated by crystallographic permutations of the structure coordinates, fractionalization of the structure coordinates, integer additions or subtractions to sets of the structure coordinates, inversion of the structure coordinates or any

15   combination of the above. Alternatively, modifications in the crystal structure due to mutations, additions, substitutions, and/or deletions of amino acids, or other changes in any of the components that make up the crystal, could also yield variations in structure coordinates. Such slight variations in the individual coordinates will have little effect on overall shape. If such variations are within an

20   acceptable standard error as compared to the original coordinates, the resulting three-dimensional shape is considered to be structurally equivalent. Structural equivalence is described in more detail below.

It should be noted that slight variations in individual structure coordinates of the human beta secretase would not be expected to significantly alter

25   the nature of chemical entities such as ligands that could associate with the inhibitor binding pockets. In this context, the phrase "associating with" refers to a condition of proximity between a chemical entity, or portions thereof, and an human beta secretase molecule or portions thereof. The association may be non-covalent, wherein the juxtaposition is energetically favored by hydrogen bonding, van der

Waals forces, or electrostatic interactions, or it may be covalent.

Thus, for example, a ligand that bound to a inhibitor binding pocket of human beta secretase would also be expected to bind to or interfere with another

5   inhibitor binding pocket whose structure coordinates define a shape that falls within the acceptable error.

It will be readily apparent to those of skill in the art that the numbering of amino acids in other isoforms of human beta secretase may be different than that of human beta secretase expressed in CHO or HKE 293 cells.

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Active Site and Other Structural Features

Applicants' invention provides information about the shape and structure of the inhibitor binding pocket of human beta secretase in the presence of a peptide mimetic inhibitor. The secondary structure of the human beta secretase

15   monomer includes two domains consistent with a typical aspartic protease fold.

Binding pockets are of significant utility in fields such as drug discovery. The association of natural ligands or substrates with the binding pockets of their corresponding receptors or enzymes is the basis of many biological mechanisms of action. Similarly, many drugs exert their biological effects through

20   association with the binding pockets of receptors and enzymes. Such associations may occur with all or any parts of the binding pocket. An understanding of such associations helps lead to the design of drugs having more favorable associations with their target, and thus improved biological effects. Therefore, this information is valuable in designing potential inhibitors of beta secretase-like inhibitor binding

25   pockets, as discussed in more detail below.

The term "binding pocket," as used herein, refers to a region of a molecule or molecular complex, that, as a result of its shape, favorably associates with another chemical entity. Thus, a binding pocket may include or consist of features such as cavities, surfaces, or interfaces between domains. Chemical entities

7

that may associate with a binding pocket include, but are not limited to, cofactors, substrates, inhibitors, agonists, and antagonists.

The amino acid constituents of an human beta secretase inhibitor

5   binding pocket as defined herein are positioned in three dimensions. In one aspect, the structure coordinates defining a inhibitor binding pocket of human beta secretase include structure coordinates of all atoms in the constituent amino acids; in another aspect, the structure coordinates of a inhibitor binding pocket include structure coordinates of just the backbone atoms of the constituent atoms.

10   The inhibitor binding pocket of human beta secretase preferably includes the amino acids listed in Table 1, more preferably the amino acids listed in Table 2, and most preferably the amino acids listed in Table 3. Alternatively, the inhibitor binding pocket of human beta secretase may be defined by those amino acids whose backbone atoms are situated within about 4 Å, more preferably within

15   about 7 Å, most preferably within about 10 Å, of one or more constituent atoms of a bound substrate or inhibitor. In yet another alternative, the inhibitor binding pocket may be defined by those amino acids whose backbone atoms are situated within a sphere centered on the coordinates representing the alpha carbon atom of residue Thr 231, the sphere having a radius of about 15 Å, preferably about 20 Å, and more

20   preferably about 25 Å.

The term "beta secretase-like inhibitor binding pocket" refers to a portion of a molecule or molecular complex whose shape is sufficiently similar to at least a portion of a inhibitor binding pocket of human beta secretase as to be expected to bind related structural analogues. A structurally equivalent inhibitor

25   binding pocket is defined by a root mean square deviation from the structure coordinates of the backbone atoms of the amino acids that make up inhibitor binding pockets in human beta secretase of at most about 0.35 Å. How this calculation is obtained is described below.

Accordingly, the invention provides molecules or molecular

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complexes comprising an human beta secretase inhibitor binding pocket or beta secretase-like inhibitor binding pocket, as defined by the sets of structure coordinates described above.

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Table 1. Residues with 4Å of inhibitor binding site.

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      GLY  11
      GLY  13
10    LEU  30
      ASP  32
      GLY  34
      SER  35
      PRO  70
15    TYR  71
      THR  72
      GLN  73
      PHE  108
      ILE  110
20    TRP  115
      ILE  126
      TYR  198
      ASP  228
      GLY  230
25    THR  231
      THR  232
      ARG  235
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30   Table 2. Residues with 7Å of inhibitor binding site.

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      SER  10
      GLY  11
      GLN  12
35    GLY  13
      TYR  14
      LEU  30
      VAL  31
      ASP  32
40    THR  33
      GLY  34
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SER   35
SER 36
ASN 37
5   VAL 69
PRO 70
TYR 71
THR 72
GLN 73
10   GLY 74
LYS 75
TRP 76
ASP 106
LYS 107
15   PHE 108
PHE 109
ILE 110
TRP 115
ILE 118
20   ILE 126
ALA 127
ARG 128
TYR 198
LYS 224
25   ILE 226
ASP 228
SER 229
GLY 230
THR 231
30   THR 232
ASN 233
ARG 235
SER 325
THR 329
35   VAL 332
ALA 335

Table 3. Residues with 10Å of inhibitor binding site.

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ARG   7
GLY   8
LYS   9

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	SER 10
	GLY 11
	GLN 12
5	GLY 13
	TYR 14
	TYR 15
	ILE 29
	LEU 30
10	VAL 31
	ASP 32
	THR 33
	GLY 34
	SER 35
15	SER 36
	ASN 37
	ALA 39
	TYR 68
	VAL 69
20	PRO 70
	TYR 71
	THR 72
	GLN 73
	GLY 74
25	LYS 75
	TRP 76
	GLU 77
	ILE 102
	SER 105
30	ASP 106
	LYS 107
	PHE 108
	PHE 109
	ILE 110
35	ASN 111
	SER 113
	TRP 115
	GLU 116
	GLY 117
40	ILE 118
	LEU 119
	GLY 120
	LEU 121

	ALA 122
	TYR 123
	ALA 124
5	GLU 125
	ILE 126
	ALA 127
	ARG 128
	PRO 129
10	LEU 154
	TRP 197
	TYR 198
	TYR 199
	ASP 223
15	LYS 224
	SER 225
	ILE 226
	VAL 227
	ASP 228
20	SER 229
	GLY 230
	THR 231
	THR 232
	ASN 233
25	LEU 234
	ARG 235
	ARG 307
	PHE 322
	ALA 323
30	ILE 324
	SER 325
	GLN 326
	SER 327
	SER 328
35	THR 329
	GLY 330
	THR 331
	VAL 332
	MET 333
40	GLY 334
	ALA 335
	VAL 336
	MET 338

GLU 339

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5   **Three-dimensional Configurations**

X-ray structure coordinates define a unique configuration of points in space. Those of skill in the art understand that a set of structure coordinates for protein or an protein/ligand complex, or a portion thereof, define a relative set of points that, in turn, define a configuration in three dimensions. A similar or

10   identical configuration can be defined by an entirely different set of coordinates, provided the distances and angles between coordinates remain essentially the same. In addition, a scalable configuration of points can be defined by increasing or decreasing the distances between coordinates by a scalar factor while keeping the angles essentially the same.

15   The present invention thus includes the scalable three-dimensional configuration of points derived from the structure coordinates of at least a portion of an human beta secretase molecule or molecular complex, as well as structurally equivalent configurations, as described below. Preferably, the scalable three-dimensional configuration includes points derived from structure coordinates

20   representing the locations of a plurality of the amino acids defining an human beta secretase inhibitor binding pocket.

In one embodiment, the scalable three-dimensional configuration includes points derived from structure coordinates representing the locations the backbone atoms of a plurality of amino acids defining the human beta secretase

25   inhibitor binding pocket, preferably the amino acids listed in Table 1, more preferably the amino acids listed in Table 2, and most preferably the amino acids listed in Table 3. Alternatively, the scalable three-dimensional configuration includes points derived from structure coordinates representing the locations of the side chain and the backbone atoms (other than hydrogens) of a plurality of the amino

30   acids defining the human beta secretase inhibitor binding pocket, preferably the

amino acids listed in Table 1, more preferably the amino acids listed in Table 2, and most preferably the amino acids listed in Table 3.

5   Likewise, the invention also includes the scalable three-dimensional configuration of points derived from structure coordinates of molecules or molecular complexes that are structurally homologous to beta secretase, as well as structurally equivalent configurations. Structurally homologous molecules or molecular complexes are defined below. Advantageously, structurally homologous molecules can be identified using the structure coordinates of human beta secretase according

10   to a method of the invention.

The configurations of points in space derived from structure coordinates according to the invention can be visualized as, for example, a holographic image, a stereodiagram, a model or a computer-displayed image, and the invention thus includes such images, diagrams or models.

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Structurally Equivalent Crystal Structures

Various computational analyses can be used to determine whether a molecule or a inhibitor binding pocket portion thereof is "structurally equivalent," defined in terms of its three-dimensional structure, to all or part of human beta

20   secretase or its inhibitor binding pockets. Such analyses may be carried out in current software applications, such as the Molecular Similarity application of QUANTA (Molecular Simulations Inc., San Diego, CA) version 4.1, and as described in the accompanying User's Guide.

The Molecular Similarity application permits comparisons between

25   different structures, different conformations of the same structure, and different parts of the same structure. The procedure used in Molecular Similarity to compare structures is divided into four steps: (1) load the structures to be compared; (2) define the atom equivalences in these structures; (3) perform a fitting operation; and (4) analyze the results.

Each structure is identified by a name. One structure is identified as the target (i.e., the fixed structure); all remaining structures are working structures (i.e., moving structures). Since atom equivalency within QUANTA is defined by

5   user input, for the purpose of this invention equivalent atoms are defined as protein backbone atoms (N, Cα, C, and O) for all conserved residues between the two structures being compared. A conserved residue is defined as a residue which is structurally or functionally equivalent. Only rigid fitting operations are considered.

When a rigid fitting method is used, the working structure is

10   translated and rotated to obtain an optimum fit with the target structure. The fitting operation uses an algorithm that computes the optimum translation and rotation to be applied to the moving structure, such that the root mean square difference of the fit over the specified pairs of equivalent atom is an absolute minimum. This number, given in angstroms, is reported by QUANTA.

15   For the purpose of this invention, any molecule or molecular complex or inhibitor binding pocket thereof, or any portion thereof, that has a root mean square deviation of conserved residue backbone atoms (N, Cα, C, O) of less than about 0.35Å, when superimposed on the relevant backbone atoms is considered "structurally equivalent" to the reference molecule. That is to say, the crystal

20   structures of those portions of the two molecules are substantially identical, within acceptable error. Particularly preferred structurally equivalent molecules or molecular complexes are those that are defined by the entire set of structure coordinates ± a root mean square deviation from the conserved backbone atoms of those amino acids of not more than 0.35 Å. More preferably, the root mean square

25   deviation is less than about 0.2 Å. Another embodiment of this invention is a molecular complex for those amino acids listed in Table 1, ± a root mean square deviation from the conserved backbone atoms of those amino acids of not more than 0.35 Å, preferably less than about 0.2 Å . Still another embodiment of this invention is a molecular complex for those amino acids listed in Table 2, ± a root

mean square deviation from the conserved backbone atoms of those amino acids of not more than 0.35 Å, preferably less than about 0.2 Å.

5 The term "root mean square deviation" means the square root of the arithmetic mean of the squares of the deviations. It is a way to express the deviation or variation from a trend or object. For purposes of this invention, the "root mean square deviation" defines the variation in the backbone of a protein from the backbone of human beta secretase or a inhibitor binding pocket portion thereof, as defined by the structure coordinates of human beta secretase described herein.

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Machine Readable Storage Media

Transformation of the structure coordinates for all or a portion of human beta secretase or the human beta secretase/ligand complex or one of its inhibitor binding pockets, for structurally homologous molecules as defined below,

15 or for the structural equivalents of any of these molecules or molecular complexes as defined above, into three-dimensional graphical representations of the molecule or complex can be conveniently achieved through the use of commercially-available software.

The invention thus further provides a machine-readable storage

20 medium comprising a data storage material encoded with machine readable data which, when using a machine programmed with instructions for using said data, is capable of displaying a graphical three-dimensional representation of any of the molecule or molecular complexes of this invention that have been described above. In a preferred embodiment, the machine-readable data storage medium comprises a

25 data storage material encoded with machine readable data which, when using a machine programmed with instructions for using said data, is capable of displaying a graphical three-dimensional representation of a molecule or molecular complex comprising all or any parts of an human beta secretase inhibitor binding pocket or an beta secretase-like inhibitor binding pocket, as defined above. In another preferred

embodiment, the machine-readable data storage medium comprises a data storage material encoded with machine readable data which, when using a machine programmed with instructions for using said data, is capable of displaying a

5   graphical three-dimensional representation of a molecule or molecular complex ± a root mean square deviation from the backbone atoms of said amino acids of not more than 0.43 Å.

In an alternative embodiment, the machine-readable data storage medium comprises a data storage material encoded with a first set of machine readable data which comprises the Fourier transform of structure coordinates, and

10   which, when using a machine programmed with instructions for using said data, can be combined with a second set of machine readable data comprising the x-ray diffraction pattern of a molecule or molecular complex to determine at least a portion of the structure coordinates corresponding to the second set of machine

15   readable data.

For example, a system for reading a data storage medium may include a computer comprising a central processing unit ("CPU"), a working memory which may be, e.g., RAM (random access memory) or "core" memory, mass storage memory (such as one or more disk drives or CD-ROM drives), one or

20   more display devices (e.g., cathode-ray tube ("CRT") displays, light emitting diode ("LED") displays, liquid crystal displays ("LCDs"), electroluminescent displays, vacuum fluorescent displays, field emission displays ("FEDs"), plasma displays, projection panels, etc.), one or more user input devices (e.g., keyboards, microphones, mice, track balls, touch pads, etc.), one or more input lines, and one or

25   more output lines, all of which are interconnected by a conventional bidirectional system bus. The system may be a stand-alone computer, or may be networked (e.g., through local area networks, wide area networks, intranets, extranets, or the internet) to other systems (e.g., computers, hosts, servers, etc.). The system may also include additional computer controlled devices such as consumer electronics and appliances.

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Input hardware may be coupled to the computer by input lines and may be implemented in a variety of ways. Machine-readable data of this invention may be inputted via the use of a modem or modems connected by a telephone line or

5   dedicated data line. Alternatively or additionally, the input hardware may comprise CD-ROM drives or disk drives. In conjunction with a display terminal, a keyboard may also be used as an input device.

Output hardware may be coupled to the computer by output lines and may similarly be implemented by conventional devices. By way of example, the

10   output hardware may include a display device for displaying a graphical representation of a binding pocket of this invention using a program such as QUANTA as described herein. Output hardware might also include a printer, so that hard copy output may be produced, or a disk drive, to store system output for later use.

15   In operation, a CPU coordinates the use of the various input and output devices, coordinates data accesses from mass storage devices, accesses to and from working memory, and determines the sequence of data processing steps. A number of programs may be used to process the machine-readable data of this invention. Such programs are discussed in reference to the computational methods

20   of drug discovery as described herein. References to components of the hardware system are included as appropriate throughout the following description of the data storage medium.

Machine-readable storage devices useful in the present invention include, but are not limited to, magnetic devices, electrical devices, optical devices,

25   and combinations thereof. Examples of such data storage devices include, but are not limited to, hard disk devices, CD devices, digital video disk devices, floppy disk devices, removable hard disk devices, magneto-optic disk devices, magnetic tape devices, flash memory devices, bubble memory devices, holographic storage devices, and any other mass storage peripheral device. It should be understood that

these storage devices include necessary hardware (e.g., drives, controllers, power supplies, etc.) as well as any necessary media (e.g., disks, flash cards, etc.) to enable the storage of data.

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Structurally Homologous Molecules, Molecular Complexes, and Crystal Structures

Structure coordinates can be used to aid in obtaining structural information about another crystallized molecule or molecular complex. The method of the invention allows determination of at least a portion of the three-dimensional structure of molecules or molecular complexes which contain one or more structural features that are similar to structural features of human beta secretase. These molecules are referred to herein as "structurally homologous" to human beta secretase. Similar structural features can include, for example, regions of amino acid identity, conserved active site or binding site motifs, and similarly arranged secondary structural elements (e.g., α helices and β sheets). Optionally, structural homology is determined by aligning the residues of the two amino acid sequences to optimize the number of identical amino acids along the lengths of their sequences; gaps in either or both sequences are permitted in making the alignment in order to optimize the number of identical amino acids, although the amino acids in each sequence must nonetheless remain in their proper order. Preferably, two amino acid sequences are compared using the Blastp program, version 2.0.9, of the BLAST 2 search algorithm, as described by Tatiana et al., FEMS Microbiol Lett 174, 247-50 (1999), and available at http://www.ncbi.nlm.nih.gov/gorf/bl2.html. Preferably, the default values for all BLAST 2 search parameters are used, including matrix = BLOSUM62; open gap penalty = 11, extension gap penalty = 1, gap x_dropoff = 50, expect = 10, wordsize = 3, and filter on. In the comparison of two amino acid sequences using the BLAST search algorithm, structural similarity is referred to as "identity." Preferably, a structurally homologous molecule is a protein that has an

amino acid sequence sharing at least 65% identity with a native or recombinant amino acid sequence of human beta secretase (for example, SEQ ID NO:1). More preferably, a protein that is structurally homologous to human beta secretase

5   includes at least one contiguous stretch of at least 50 amino acids that shares at least 80% amino acid sequence identity with the analogous portion of the native or recombinant human beta secretase (for example, SEQ ID NO:1). Methods for generating structural information about the structurally homologous molecule or molecular complex are well-known and include, for example, molecular

10   replacement techniques.

Therefore, in another embodiment this invention provides a method of utilizing molecular replacement to obtain structural information about a molecule or molecular complex whose structure is unknown comprising the steps of:

(a) crystallizing the molecule or molecular complex of unknown

15   structure;

(b) generating an x-ray diffraction pattern from said crystallized molecule or molecular complex; and

(c) applying at least a portion of the structure to the x-ray diffraction pattern to generate a three-dimensional electron density map of the molecule or

20   molecular complex whose structure is unknown.

By using molecular replacement, all or part of the structure coordinates of human beta secretase or the human beta secretase/ligand complex as provided by this invention can be used to determine the structure of a crystallized molecule or molecular complex whose structure is unknown more quickly and

25   efficiently than attempting to determine such information *ab initio*.

Molecular replacement provides an accurate estimation of the phases for an unknown structure. Phases are a factor in equations used to solve crystal structures that cannot be determined directly. Obtaining accurate values for the phases, by methods other than molecular replacement, is a time-consuming process

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that involves iterative cycles of approximations and refinements and greatly hinders

the solution of crystal structures. However, when the crystal structure of a protein

containing at least a structurally homologous portion has been solved, the phases

5   from the known structure provide a satisfactory estimate of the phases for the

unknown structure.

Thus, this method involves generating a preliminary model of a

molecule or molecular complex whose structure coordinates are unknown, by

orienting and positioning the relevant portion of human beta secretase or the human

10   beta secretase/inhibitor complex within the unit cell of the crystal of the unknown

molecule or molecular complex so as best to account for the observed x-ray

diffraction pattern of the crystal of the molecule or molecular complex whose

structure is unknown. Phases can then be calculated from this model and combined

with the observed x-ray diffraction pattern amplitudes to generate an electron

15   density map of the structure whose coordinates are unknown. This, in turn, can be

subjected to any well-known model building and structure refinement techniques to

provide a final, accurate structure of the unknown crystallized molecule or

molecular complex (E. Lattman, "Use of the Rotation and Translation Functions," in

Meth. Enzymol., 115, pp. 55-77 (1985); M.G. Rossman, ed., "The Molecular

20   Replacement Method," Int. Sci. Rev. Ser., No. 13, Gordon & Breach, New York

(1972)).

Structural information about a portion of any crystallized molecule or

molecular complex that is sufficiently structurally homologous to a portion of

human beta secretase can be resolved by this method. In addition to a molecule that

25   shares one or more structural features with human beta secretase as described above,

a molecule that has similar bioactivity, such as the same catalytic activity, substrate

specificity or ligand binding activity as human beta secretase, may also be

sufficiently structurally homologous to human beta secretase to permit use of the

structure coordinates of human beta secretase to solve its crystal structure.

In a preferred embodiment, the method of molecular replacement is utilized to obtain structural information about a molecule or molecular complex, wherein the molecule or molecular complex comprises at least one human beta

5 secretase subunit or homolog. A "subunit" of human beta secretase is an human beta secretase molecule that has been truncated at the N-terminus or the C-terminus, or both. In the context of the present invention, a "homolog" of human beta secretase is a protein that contains one or more amino acid substitutions, deletions, additions, or rearrangements with respect to the amino acid sequence of human beta

10 secretase (SEQ ID NO:1), but that, when folded into its native conformation, exhibits or is reasonably expected to exhibit at least a portion of the tertiary (three-dimensional) structure of human beta secretase. For example, structurally homologous molecules can contain deletions or additions of one or more contiguous or noncontiguous amino acids, such as a loop or a domain. Structurally homologous

15 molecules also include "modified" human beta secretase molecules that have been chemically or enzymatically derivatized at one or more constituent amino acid, including side chain modifications, backbone modifications, and N- and C- terminal modifications including acetylation, hydroxylation, methylation, amidation, and the attachment of carbohydrate or lipid moieties, cofactors, and the like.

20 A heavy atom derivative of human beta secretase is also included as an human beta secretase homolog. The term "heavy atom derivative" refers to derivatives of human beta secretase produced by chemically modifying a crystal of human beta secretase. In practice, a crystal is soaked in a solution containing heavy metal atom salts, or organometallic compounds, e.g., lead chloride, gold thiomalate,

25 thiomersal or uranyl acetate, which can diffuse through the crystal and bind to the surface of the protein. The location(s) of the bound heavy metal atom(s) can be determined by x-ray diffraction analysis of the soaked crystal. This information, in turn, is used to generate the phase information used to construct three-dimensional structure of the protein (T.L. Blundell and N.L. Johnson, Protein Crystallography,

22

Academic Press (1976)).

Because human beta secretase can crystallize in more than one crystal form, the structure coordinates of human beta secretase as provided by this invention

5   are particularly useful in solving the structure of other crystal forms of human beta secretase or human beta secretase complexes.

The structure coordinates of human beta secretase as provided by this invention are particularly useful in solving the structure of human beta secretase mutants. Mutants may be prepared, for example, by expression of human beta

10   secretase cDNA previously altered in its coding sequence by oligonucleotide-directed mutagenesis. Mutants may also be generated by site-specific incorporation of unnatural amino acids into beta secretase proteins using the general biosynthetic method of C.J. Noren et al., Science, 244:182-188 (1989). In this method, the codon encoding the amino acid of interest in wild-type human beta secretase is replaced by

15   a "blank" nonsense codon, TAG, using oligonucleotide-directed mutagenesis. A suppressor tRNA directed against this codon is then chemically aminoacylated *in vitro* with the desired unnatural amino acid. The aminoacylated tRNA is then added to an *in vitro* translation system to yield a mutant human beta secretase with the site-specific incorporated unnatural amino acid.

20   Selenocysteine or selenomethionine may be incorporated into wild-type or mutant human beta secretase by expression of human beta secretase-encoding cDNAs in auxotrophic *E. coli* strains (W.A. Hendrickson et al., EMBO J., 9(5):1665-1672 (1990)). In this method, the wild-type or mutagenized human beta secretase cDNA may be expressed in a host organism on a growth medium depleted

25   of either natural cysteine or methionine (or both) but enriched in selenocysteine or selenomethionine (or both). Alternatively, selenomethionine analogues may be prepared by down regulation methionine biosynthesis. (T.E. Benson et al., Nat. Struct. Biol., 2:644-53 (1995); G.D. Van Duyne et al., J. Mol. Biol. 229:105-24 (1993)).

The structure coordinates of human beta secretase are also particularly useful to solve the structure of crystals of human beta secretase, human beta secretase mutants or human beta secretase homologs co-complexed with a

5   variety of chemical entities. This approach enables the determination of the optimal sites for interaction between chemical entities, including candidate human beta secretase inhibitors and human beta secretase. Potential sites for modification within the various binding site of the molecule can also be identified. This information provides an additional tool for determining the most efficient binding

10   interactions, for example, increased hydrophobic interactions, between human beta secretase and a chemical entity. For example, high resolution x-ray diffraction data collected from crystals exposed to different types of solvent allows the determination of where each type of solvent molecule resides. Small molecules that bind tightly to those sites can then be designed and synthesized and tested for their

15   human beta secretase inhibition activity.

All of the complexes referred to above may be studied using well-known x-ray diffraction techniques and may be refined versus 1.5-3 Å resolution x-ray data to an R value of about 0.20 or less using computer software, such as X-PLOR (Yale University, 81992, distributed by Molecular Simulations, Inc.; see, e.g.,

20   Blundell & Johnson, supra; Meth. Enzymol., Vol. 114 & 115, H.W. Wyckoff et al., eds., Academic Press (1985)). This information may thus be used to optimize known human beta secretase inhibitors, and more importantly, to design new human beta secretase inhibitors.

The invention also includes the unique three-dimensional

25   configuration defined by a set of points defined by the structure coordinates for a molecule or molecular complex structurally homologous to human beta secretase as determined using the method of the present invention, structurally equivalent configurations, and magnetic storage media comprising such set of structure coordinates.

24

Further, the invention includes structurally homologous molecules as identified using the method of the invention.

5   **Homology Modeling**

Using homology modeling, a computer model of an human beta secretase homolog can be built or refined without crystallizing the homolog. First, a preliminary model of the human beta secretase homolog is created by sequence alignment with human beta secretase, secondary structure prediction, the screening

10   of structural libraries, or any combination of those techniques. Computational software may be used to carry out the sequence alignments and the secondary structure predictions. Structural incoherences, e.g., structural fragments around insertions and deletions, can be modeled by screening a structural library for peptides of the desired length and with a suitable conformation. For prediction of

15   the side chain conformation, a side chain rotamer library may be employed. If the human beta secretase homolog has been crystallized, the final homology model can be used to solve the crystal structure of the homolog by molecular replacement, as described above. Next, the preliminary model is subjected to energy minimization to yield an energy minimized model. The energy minimized model may contain

20   regions where stereochemistry restraints are violated, in which case such regions are remodeled to obtain a final homology model. The homology model is positioned according to the results of molecular replacement, and subjected to further refinement comprising molecular dynamics calculations.

25   **Rational Drug Design**

Computational techniques can be used to screen, identify, select and/or design chemical entities capable of associating with human beta secretase or structurally homologous molecules. Knowledge of the structure coordinates for human beta secretase permits the design and/or identification of synthetic

compounds and/or other molecules which have a shape complementary to the

conformation of the human beta secretase binding site. In particular, computational

techniques can be used to identify or design chemical entities, such as inhibitors,

5   agonists and antagonists, that associate with an human beta secretase inhibitor

binding pocket or an beta secretase-like inhibitor binding pocket. Inhibitors may

bind to or interfere with all or a portion of an active site of human beta secretase,

and can be competitive, non-competitive, or uncompetitive inhibitors; or interfere

with dimerization by binding at the interface between the two monomers. Once

10   identified and screened for biological activity, these inhibitors/agonists/antagonists

may be used therapeutically or prophylactically to block human beta secretase

activity and, thus, inhibit the growth of the bacteria or cause its death. Structure-

activity data for analogues of ligands that bind to or interfere with human beta

secretase or beta secretase-like inhibitor binding pockets can also be obtained

15   computationally.

The term "chemical entity," as used herein, refers to chemical

compounds, complexes of two or more chemical compounds, and fragments of such

compounds or complexes. Chemical entities that are determined to associate with

human beta secretase are potential drug candidates.

20   Data stored in a machine-readable storage medium that is capable of

displaying a graphical three-dimensional representation of the structure of human

beta secretase or a structurally homologous molecule, as identified herein, or

portions thereof may thus be advantageously used for drug discovery. The structure

coordinates of the chemical entity are used to generate a three-dimensional image

25   that can be computationally fit to the three-dimensional image of human beta

secretase or a structurally homologous molecule. The three-dimensional molecular

structure encoded by the data in the data storage medium can then be

computationally evaluated for its ability to associate with chemical entities. When

the molecular structures encoded by the data is displayed in a graphical three-

dimensional representation on a computer screen, the protein structure can also be visually inspected for potential association with chemical entities.

One embodiment of the method of drug design involves evaluating
5   the potential association of a known chemical entity with human beta secretase or a structurally homologous molecule, particularly with an human beta secretase inhibitor binding pocket or beta secretase-like inhibitor binding pocket. The method of drug design thus includes computationally evaluating the potential of a selected chemical entity to associate with any of the molecules or molecular complexes set
10   forth above. This method comprises the steps of: (a) employing computational means to perform a fitting operation between the selected chemical entity and a inhibitor binding pocket or a pocket nearby the inhibitor binding pocket of the molecule or molecular complex; and (b) analyzing the results of said fitting operation to quantify the association between the chemical entity and the inhibitor
15   binding pocket.

In another embodiment, the method of drug design involves computer-assisted design of chemical entities that associate with human beta secretase, its homologs, or portions thereof. Chemical entities can be designed in a step-wise fashion, one fragment at a time, or may be designed as a whole or "de
20   novo."

To be a viable drug candidate, the chemical entity identified or designed according to the method must be capable of structurally associating with at least part of an human beta secretase or beta secretase-like inhibitor binding pockets, and must be able, sterically and energetically, to assume a conformation that allows
25   it to associate with the human beta secretase or beta secretase-like inhibitor binding pocket. Non-covalent molecular interactions important in this association include hydrogen bonding, van der Waals interactions, hydrophobic interactions, and electrostatic interactions. Conformational considerations include the overall three-dimensional structure and orientation of the chemical entity in relation to the

inhibitor binding pocket, and the spacing between various functional groups of an entity that directly interact with the beta secretase-like inhibitor binding pocket or homologs thereof.

5   Optionally, the potential binding of a chemical entity to an human beta secretase or beta secretase-like inhibitor binding pocket is analyzed using computer modeling techniques prior to the actual synthesis and testing of the chemical entity. If these computational experiments suggest insufficient interaction and association between it and the human beta secretase or beta secretase-like

10   inhibitor binding pocket, testing of the entity is obviated. However, if computer modeling indicates a strong interaction, the molecule may then be synthesized and tested for its ability to bind to or interfere with an human beta secretase or beta secretase-like inhibitor binding pocket. Binding assays to determine if a compound actually interferes with human beta secretase can also be performed and are well

15   known in the art. Binding assays may employ kinetic or thermodynamic methodology using a wide variety of techniques including, but not limited to, microcalorimetry, circular dichroism, capillary zone electrophoresis, nuclear magnetic resonance spectroscopy, fluorescence spectroscopy, and combinations thereof.

20   One skilled in the art may use one of several methods to screen chemical entities or fragments for their ability to associate with an human beta secretase or beta secretase-like inhibitor binding pocket. This process may begin by visual inspection of, for example, an human beta secretase or beta secretase-like inhibitor binding pocket on the computer screen based on the human beta secretase

25   structure coordinates or other coordinates which define a similar shape generated from the machine-readable storage medium. Selected fragments or chemical entities may then be positioned in a variety of orientations, or docked, within the inhibitor binding pocket. Docking may be accomplished using software such as QUANTA

and SYBYL, followed by energy minimization and molecular dynamics with standard molecular mechanics forcefields, such as CHARMM and AMBER.

Specialized computer programs may also assist in the process of

5 selecting fragments or chemical entities. Examples include GRID (P.J. Goodford, J. Med. Chem. 28:849-857 (1985); available from Oxford University, Oxford, UK); MCSS (A. Miranker et al., Proteins: Struct. Funct. Gen.,11:29-34 (1991); available from Molecular Simulations, San Diego, CA); AUTODOCK (D.S. Goodsell et al., Proteins: Struct. Funct. Genet. 8:195-202 (1990); available from Scripps Research

10 Institute, La Jolla, CA); and DOCK (I.D. Kuntz et al., J. Mol. Biol. 161:269-288 (1982); available from University of California, San Francisco, CA).

Once suitable chemical entities or fragments have been selected, they can be assembled into a single compound or complex. Assembly may be preceded by visual inspection of the relationship of the fragments to each other on the three-

15 dimensional image displayed on a computer screen in relation to the structure coordinates of human beta secretase. This would be followed by manual model building using software such as QUANTA or SYBYL (Tripos Associates, St. Louis, MO).

Useful programs to aid one of skill in the art in connecting the

20 individual chemical entities or fragments include, without limitation, CAVEAT (P.A. Bartlett et al., in Molecular Recognition in Chemical and Biological Problems," Special Publ., Royal Chem. Soc., 78:182-196 (1989); G. Lauri et al., J. Comput. Aided Mol. Des. 8:51-66 (1994); available from the University of California, Berkeley, CA); 3D database systems such as ISIS (available from MDL

25 Information Systems, San Leandro, CA; reviewed in Y.C. Martin, J. Med. Chem. 35:2145-2154 (1992)); and HOOK (M.B. Eisen et al., Proteins: Struc., Funct., Genet. 19:199-221 (1994); available from Molecular Simulations, San Diego, CA).

Human beta secretase binding compounds may be designed "de novo" using either an empty binding site or optionally including some portion(s) of a

known inhibitor(s). There are many *de novo* ligand design methods including, without limitation, LUDI (H.-J. Bohm, J. Comp. Aid. Molec. Design. 6:61-78 (1992); available from Molecular Simulations Inc., San Diego, CA); LEGEND (Y.

5   Nishibata et al., Tetrahedron, 47:8985 (1991); available from Molecular Simulations Inc., San Diego, CA); LeapFrog (available from Tripos Associates, St. Louis, MO); and SPROUT (V. Gillet et al., J. Comput. Aided Mol. Design 7:127-153 (1993); available from the University of Leeds, UK).

Once a compound has been designed or selected by the above

10   methods, the efficiency with which that entity may bind to or interfere with an human beta secretase or beta secretase-like inhibitor binding pocket may be tested and optimized by computational evaluation. For example, an effective human beta secretase or beta secretase-like inhibitor binding pocket inhibitor must preferably demonstrate a relatively small difference in energy between its bound and free states

15   (i.e., a small deformation energy of binding). Thus, the most efficient human beta secretase or beta secretase-like inhibitor binding pocket inhibitors should preferably be designed with a deformation energy of binding of not greater than about 10 kcal/mole; more preferably, not greater than 7 kcal/mole. human beta secretase or beta secretase-like inhibitor binding pocket inhibitors may interact with the inhibitor

20   binding pocket in more than one conformation that is similar in overall binding energy. In those cases, the deformation energy of binding is taken to be the difference between the energy of the free entity and the average energy of the conformations observed when the inhibitor binds to the protein.

An entity designed or selected as binding to or interfering with an

25   human beta secretase or beta secretase-like inhibitor binding pocket may be further computationally optimized so that in its bound state it would preferably lack repulsive electrostatic interaction with the target enzyme and with the surrounding water molecules. Such non-complementary electrostatic interactions include repulsive charge-charge, dipole-dipole, and charge-dipole interactions.

Specific computer software is available in the art to evaluate compound deformation energy and electrostatic interactions. Examples of programs designed for such uses include: Gaussian 94, revision C (M.J. Frisch, Gaussian, Inc., Pittsburgh, PA 81995); AMBER, version 4.1 (P.A. Kollman, University of California at San Francisco, 81995); QUANTA/CHARMM (Molecular Simulations, Inc., San Diego, CA 81995); Insight II/Discover (Molecular Simulations, Inc., San Diego, CA 81995); DelPhi (Molecular Simulations, Inc., San Diego, CA 81995); and AMSOL (Quantum Chemistry Program Exchange, Indiana University). These programs may be implemented, for instance, using a Silicon Graphics workstation such as an Indigo^2 with "IMPACT" graphics. Other hardware systems and software packages will be known to those skilled in the art.

Another approach encompassed by this invention is the computational screening of small molecule databases for chemical entities or compounds that can bind in whole, or in part, to a human beta secretase or beta secretase-like inhibitor binding pocket. In this screening, the quality of fit of such entities to the binding site may be judged either by shape complementarity or by estimated interaction energy (E.C. Meng et al., J. Comp. Chem., 13, pp. 505-524 (1992)).

This invention also enables the development of chemical entities that can isomerize to short-lived reaction intermediates in the chemical reaction of a substrate or other compound that interferes with or with human beta secretase. Time-dependent analysis of structural changes in human beta secretase during its interaction with other molecules is carried out. The reaction intermediates of human beta secretase can also be deduced from the reaction product in co-complex with human beta secretase. Such information is useful to design improved analogues of known human beta secretase inhibitors or to design novel classes of inhibitors based on the reaction intermediates of the human beta secretase and inhibitor co-complex. This provides a novel route for designing human beta secretase inhibitors with both

high specificity and stability.

Yet another approach to rational drug design involves probing the human beta secretase crystal of the invention with molecules comprising a variety of

5   different functional groups to determine optimal sites for interaction between candidate human beta secretase inhibitors and the protein. For example, high resolution x-ray diffraction data collected from crystals soaked in or co-crystallized with other molecules allows the determination of where each type of solvent molecule sticks. Molecules that bind tightly to those sites can then be further

10   modified and synthesized and tested for their beta secretase inhibitor activity (J. Travis, Science, 262:1374 (1993)).

In a related approach, iterative drug design is used to identify inhibitors of human beta secretase. Iterative drug design is a method for optimizing associations between a protein and a compound by determining and evaluating the

15   three-dimensional structures of successive sets of protein/compound complexes. In iterative drug design, crystals of a series of protein/compound complexes are obtained and then the three-dimensional structures of each complex is solved. Such an approach provides insight into the association between the proteins and compounds of each complex. This is accomplished by selecting compounds with

20   inhibitory activity, obtaining crystals of this new protein/compound complex, solving the three dimensional structure of the complex, and comparing the associations between the new protein/compound complex and previously solved protein/compound complexes. By observing how changes in the compound affected the protein/compound associations, these associations may be optimized.

25   A compound that is identified or designed as a result of any of these methods can be obtained (or synthesized) and tested for its biological activity, e.g., inhibition of beta secretase activity.

Pharmaceutical Compositions (Inhibitors)

Pharmaceutical compositions of this invention comprise an inhibitor of human beta secretase activity identified according to the invention, or a

5   pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier, adjuvant, or vehicle. The term "pharmaceutically acceptable carrier" refers to a carrier(s) that is "acceptable" in the sense of being compatible with the other ingredients of a composition and not deleterious to the recipient thereof. Optionally, the pH of the formulation is adjusted with pharmaceutically acceptable acids, bases,

10   or buffers to enhance the stability of the formulated compound or its delivery form.

Methods of making and using such pharmaceutical compositions are also included in the invention. The pharmaceutical compositions of the invention can be administered orally, parenterally, by inhalation spray, topically, rectally, nasally, buccally, vaginally, or via an implanted reservoir. Oral administration or

15   administration by injection is preferred. The term parenteral as used herein includes subcutaneous, intracutaneous, intravenous, intramuscular, intra-articular, intrasynovial, intrasternal, intrathecal, intralesional, and intracranial injection or infusion techniques.

Dosage levels of between about 0.01 and about 100 mg/kg body

20   weight per day, preferably between about 0.5 and about 75 mg/kg body weight per day of the human beta secretase inhibitory compounds described herein are useful for the prevention and treatment of human beta secretase mediated disease. Typically, the pharmaceutical compositions of this invention will be administered from about 1 to about 5 times per day or alternatively, as a continuous infusion.

25   Such administration can be used as a chronic or acute therapy. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. A typical preparation will contain from about 5% to about 95%

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active compound (w/w). Preferably, such preparations contain from about 20% to about 80% active compound.

In order that this invention be more fully understood, the following

5   examples are set forth. These examples are for the purpose of illustration only and are not to be construed as limiting the scope of the invention in any way.

EXAMPLES

10

Crystallization and Structure Determination of Human Beta Secretase

A. Expression, Purification, and Crystallization

Expression and Purification of Beta Secretase from HEK 293 Cells.

The expression plasmid = pcDNA3.1/myc/his (neomycin)

15   (Invitrogen) contains beta secretase extending from Met [-21] to Ser [432] with a myc tag followed by a hexahistidine tag [EQKLISEEDLNMHTEHHHHHH*] at the C-terminus. Following transfection in HEK293 cells, stable cells were selected using 0.8 mg/ml G418. A stable clone of transfected HEK293 cells that secretes human beta-secretase was expanded in static, monolayer cell culture. Confluent

20   cultures were detached by shaking and a plurality of plastic, 225 cm^2 T-flasks were each inoculated with a suspension of 1-5 x 106 cells in 100 ml of High-Glucose Dulbecco's Modified Eagle medium that was supplemented with 5% fetal bovine serum and 500 micrograms/ml G418. These cell cultures were incubated in a humidified. 37°C incubator gassed with 95% air and 5% CO$_2$. Once the cells reached

25   confluence the growth medium in each flask was removed and replaced with 100 ml fresh medium. The conditioned, culture medium supernatant was harvested aseptically and replaced by fresh medium every 48-72 hours. The harvested medium was pooled, centrifuged at 1000 x g to remove cell debris, and was stored in plastic bottles at 4°C. Cell monolayers were maintained in semi-continuous culture for

several weeks until the cells either began to die or to detach from the culture flasks. The cells were then resuspended and used to inoculate a fresh set of production flasks.

5   For purification, the medium was concentrated approximately 10-fold using a tangential flow concentrator equipped with a 30,000 molecular weight cutoff cartridge. Solid ammonium sulfate was then slowly added with stirring to the concentrate at 4°C to a final value of 40% saturation (242 g/L). After stirring at 4°C for 30 minutes, the suspension was clarified by centrifugation (16,000 x g, 60

10   minutes) and the supernatant taken for further analysis. The 40% ammonium sulfate supernatant was adjusted to 80% saturation by slow addition of solid ammonium sulfate with stirring at 4°C (281 g/L). After stirring for 30 minutes at 4°C, the insoluble material was collected by centrifugation as indicated above and the 40-80% ammonium sulfate pellet taken for further analysis.

15   The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-HCI (8.5)10.5 M NaCIIIO mM imidazole (1/10 the original volume) and applied to a 12.5 ml column containing Ni^+-NTA Fast Flow resin previously equilibrated in the same buffer. Following sample application, the column was washed with 10 column volumes of loading buffer and then eluted with 25 mM Tris-HC1 (8.5)/0.5

20   M NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane (30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, PNU-292593E) was

25   synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and immediately applied to the PNU-292593E/sulfolink column (6 ml containing 1.0 mg PNU-292593/ml of resin) that had been previously equilibrated in 25 mM

NaOAc (4.5). Following sample application, the column was washed with 10 column volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM NaBO$_3$ (8.5). N-terminal sequence analysis of the affinity purified material revealed an equimolar

5   mixture of pro- and processed human beta-secretase beginning at Thr^1 and Glu^{25} respectively. The final protein concentration was determined by amino acid analysis assuming a 60 kDa glycoprotein.

Production of Recombinant Human β-Secretase in Insect sf9 Cells and CHO-K1 Cells. The coding sequence was engineered to delete the terminal

10   transmembrane and cytoplasmic domain and introduce a C-terminal hexahistidine tag using the polymerase chain reaction. The 5' sense oligonucleotide primer [CGCTTTGGATCCGTGGACAACCTGAGGGGCAA] was designed to incorporate a BamHI site for ease in subcloning and Kozak consensus sequence around the initiator methionine for optimal translation initiation. The 3' antisense

15   primer [CGCTTTGGTACCCTATGACTCATCTGTCTGTGGAATGTTG] incorporated a hexahistidine tag and translation termination codon just upstream of the predicted transmembrane domain (Ser^{432}) and a NotI restriction site for cloning. The PCR was performed on the plasmid template pcDNA3.1hygroAsp2R for 15 cycles [94°C, 30 sec., 65°C, 30 sec., 72°C, 30 sec] using Pwo I polymerase (Roche

20   Biochemicals, Indianapolis, IN) as outlined by the manufacturer. The PCR product was digested to completion with *Bam*HI and *Not*I and ligated into the *Bam*HI and *Not*I sites of the baculovirus transfer vector pVL1393 (PharMingen, San Diego, CA). A portion of the ligation was used to transform competent E. coli DH5α cells and recombinant clones were selected on ampicillin. Individual clones containing

25   the proper cDNA inserts were identified by PCR. Plasmid DNA from clone (pVL1393/Hu_Asp-2LΔTM(His)₆) was prepared by alkaline lysis and banding in CsCl. The integrity of the insert was confirmed by complete DNA sequencing. For CHO-K1 cell expression, plasmid pVL1393/Hu_Asp-2LΔTM(His)₆ was digested with *Bam*HI and *Not*I and the resulting fragment subcloned into the mammalian

expression vector pcDNA3.1(hygro) as described above to yield pcDNA3.1(hygro)/ Hu_Asp-2LΔTM(His)$_6$).

For expression, CHO-K1 cells (50% confluent) were transfected with

5   cationic liposome/pcDNA3.1(hygro)/ Hu_Asp-2LΔTM(His)$_6$ complexes in α-MEM medium containing 10% FBS overnight. Selection was performed in the same medium containing 0.5 mg/L hygromycin B for seven days and surviving cells were cloned by limiting dilution. Eight cell lines were screened for soluble β-secretase by Western blot analysis using a polyclonal rabbit antiserum specific for human β-

10   secretase (UP-191). Conditioned medium from each clonal cell line was concentrated by Ni$^+$-NTA chromatography and the histidine-tagged polypeptide eluted with buffer containing 50 mM imidazole. Aliquots of the latter fraction were displayed on a PVDF membrane and recombinant soluble human β-secretase was visualized using UP-191 antiserum and alkaline phosphatase conjugated goat

15   antirabbit second antibody. Based on these results, clone #4 showed the highest expression level and was used for all subsequent experiments.

For baculovirus expression, recombinant virus containing the coding sequence of soluble β-secretase was isolated following recombination of the plasmid transfer vector pVL1393/Hu_Asp-2LΔTM(His)$_6$ in sf9 cells using standard methods.

20   Individual virus isolates were used to infect sf9 cells and expression of the desired polypeptide was quantified by Western blot analysis of the conditioned medium collected 69 hr post infection as described above. The recombinant virus directing the synthesis of the highest level of β-secretase was scaled-up for protein production.

25

Purification of Recombinant Human β-Secretase from BVES or

CHO-K1 Cells. For production of soluble β-secretase in insect cells, sf9 cells were infected with recombinant baculovirus at a multiplicity of infection of 1.0 in serum free medium and conditioned medium harvested 69 hours post-infection. For CHO

cells, the production cell line expressing secreted soluble human β-secretase was expanded in either roller bottles or in a packed-bed bioreactor in medium containing 0.5 fetal bovine serum. Conditioned medium was collected and stored at 4°C until

5   processing.

For purification from either source, the medium was concentrated approximately 10-fold using a tangential flow concentrator equip with a 30,000 molecular weight cutoff cartridge. Solid ammonium sulfate was then slowly added with stirring to the concentrate at 4°C to a final value of 40% saturation (242 g/L).

10   After stirring at 4°C for 30 minutes, the suspension was clarified by centrifugation (16,000 x g, 60 minutes) and the supernatant taken for further analysis. The 40% ammonium sulfate supernatant was adjusted to 80% saturation by slow addition of solid ammonium sulfate with stirring at 4°C (281 g/L). After stirring for 30 minutes at 4°C, the insoluble material was collected by centrifugation as indicated above and

15   the 40-80% ammonium sulfate pellet taken for further analysis.

The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-HCl (8.5)/0.5 M NaCl/10 mM imidazole (1/10 the original volume) and applied to a 12.5 ml column containing Ni^+-NTA Fast Flow resin previously equilibrated in the same buffer. Following sample application, the column was washed with 10 column

20   volumes of loading buffer and then eluted with 25 mM Tris-HCl (8.5)/0.5 M NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane (30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-

25   Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, PNU-292593E) was synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and immediately applied to the PNU-292593E/sulfolink column (6 ml containing 1.0 mg

PNU-292593/ml of resin) that had been previously equilibrated in 25 mM NaOAc
(4.5). Following sample application, the column was washed with 10 column
volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM $NaBO_3$ (8.5). N-
5   terminal sequence analysis of the affinity purified material revealed an equimolar
mixture of pro- and processed human β-secretase beginning at Thr^1 and Glu^{25},
respectively. The final protein concentration was determined by amino acid analysis
assuming a 52 kDa glycoprotein for insect cells and a 60 kDa glycoprotein for CHO
cells, respectively.

10

Purification of Recombinant Human β-Secretase from CHO-K1

Cells. The production cell line expressing secreted soluble human β-secretase was
expanded in either roller bottles or in a packed-bed bioreactor in medium containing
0.5 fetal bovine serum. Conditioned medium was collected and stored at 4°C until
15   processing. For purification, the medium was concentrated approximately 10-fold
using a tangential flow concentrator equip with a 30,000 molecular weight cutoff
cartridge. Solid ammonium sulfate was then slowly added with stirring to the
concentrate at 4°C to a final value of 40% saturation (242 g/L). After stirring at 4°C
for 30 minutes, the suspension was clarified by centrifugation (16,000 x g, 60
20   minutes) and the supernatant taken for further analysis. The 40% ammonium sulfate
supernatant was adjusted to 80% saturation by slow addition of solid ammonium
sulfate with stirring at 4°C (281 g/L). After stirring for 30 minutes at 4°C, the
insoluble material was collected by centrifugation as indicated above and the 40-
80% ammonium sulfate pellet taken for further analysis.

25   The 40-80% ammonium sulfate pellet was dissolved in 25 mM Tris-
HCl (8.5)/0.5 M NaCl/10 mM imidazole (1/10 the original volume) and applied to a
12.5 ml column containing Ni^+-NTA Fast Flow resin previously equilibrated in the
same buffer. Following sample application, the column was washed with 10 column
volumes of loading buffer and then eluted with 25 mM Tris-HCl (8.5)/0.5 M

39

NaCl/50 mM imidazole. The material eluting in 50 mM imidazole was pooled, concentrated approximately 10-fold using a YM 30 membrane (30,000 MWCO), and then dialyzed against 10 mM HEPES-Na (8.0) using 50,000 molecular weight

5   cutoff tubing. For affinity purification, the synthetic peptide Ser-Glu-Val-Asn-Sta-Val-Ala-Glu-Phe-Arg-Gly-Gly-Cys (where Sta = statine, inhibitor shown in Figure 1A) was synthesized and coupled to sulfolink resin (Pierce Chemical Company) as recommended by the manufacture. The dialyzed material from above was adjusted to 0.1 M NaOAc (4.5) by addition of 1/10 volume of 1.0 M NaOAc (4.5) and

10   immediately applied to the inhibitor shown in Figure 1A/sulfolink column (6 ml containing 1.0 mg of the inhibitor shown in Figure 1A/ml of resin) that had been previously equilibrated in 25 mM NaOAc (4.5). Following sample application, the column was washed with 10 column volumes of 25 mM NaOAc (4.5) and then eluted with 50 mM $NaBO_3$ (8.5). N-terminal sequence analysis of the affinity

15   purified material revealed an equimolar mixture of pro- and processed human β-secretase beginning at Thr^1 and Glu^{25}, respectively. The final protein concentration was determined by amino acid analysis assuming a 60 kDa glycoprotein.

　　　　　　　　　Protein Crystallization – Condition #1. Initial screening of human beta secretase involved the use of commercially available screens from Hampton

20   Research, Emerald BioStructures, and an in-house proprietary screen. Screening was conducted primarily with 15 mg/ml protein with and without 400μM of the inhibitor shown in Figure 1A at 4°C and 20°C. The only positive hits from the proprietary screen at 4°C are as follows.

　　　　　　　　　The human beta secretase protein was purified from a CHO cell

25   culture (see above) and the final sample was stored in 25mM MOPS pH 7.5. This protein is highly glycosylated and contains a 50:50 mixture of pro and processed forms, 438 and 414 amino acid residues respectively. The peptide inhibitor shown in Figure 1A is dissolved in 25mM Tris pH 8.0 to a 1.67mM stock. This stock is then used to make the 15 mg/ml human beta secretase/ 400μM of the inhibitor

shown in Figure 1A solution. The conditions that give crystals are: 1) 15% PEG 10,000, 20mM ammonium sulfate, 50mM sodium acetate pH 5.0, (final pH of well solution - 4.08) at 4°C. Crystals grew within three days. 2) 10% PEG 12,000,

5   50mM sodium acetate pH 4.5, (final pH of well solution - 4.32) at 4°C. Crystals grew within three weeks. The trays are setup using a hanging drop vapor diffusion method with a 1:1 protein:well solution ratio. Both conditions give the same crystal morphology. These conditions are optimized by testing various PEG sizes and concentration, various acetate, sulfate and chloride salts, and a sodium acetate pH

10   range. The optimization of these conditions resulted in the following preferred range of conditions: 10-14% PEG 4,000 or PEG 10,000, 50mM sodium acetate pH 4.2, 20mM ammonium sulfate, 1μL well solution + 1μL protein/compound solution, hanging drop vapor diffusion setup with 500μL well solution volume, and an incubation temperature of 4°C. Crystals grow within three days with a final size of

15   0.4x0.2x0.2mm.

Moving the crystals into cryogenic conditions uses an addition method. Cryo solutions are made to mimic the contents of the crystallization drop with the addition of 5, 10, 15, and 20% ethylene glycol. Example of a final cryo solution: 13% PEG 10,000, 50mM sodium acetate pH 4.2, 25mM MOPS pH 7.5,

20   20mM ammonium sulfate, 20% ethylene glycol. The method for introducing the crystals into cryo involves a slow addition method. Small amounts of a 5% ethylene glycol cryo solution are added directly to the drop containing crystals. Eventually, through several steps, the 20% ethylene glycol solution is added directly to the drop. The drop is periodically tested for "glass" freezing characteristics. The crystals are

25   then looped out of drop and dipped directly into liquid nitrogen.

The best diffraction with the above-mentioned crystals is 6.5Å on a synchrotron radiation source (APS). Along with the limited diffraction limit, the unit cell (a=175Å, b=295Å, c=441Å, α=90°, β=91.2°, γ=90°) makes high resolution data collection impossible. These crystallization conditions have been abandoned

for this reason.

Protein Crystallization – Condition #2. Based on observations of the initial screening effort, fresh protein derived from CHO cells was concentrated to

5 42mg/ml and mixed with a new inhibitor (Figure 1B) so that the final concentration of the mix was 40mg/ml beta secretase, and 2mM of the inhibitor shown in Figure 1B, in 20mM Hepes pH 7.8, 10% DMSO. This preparation was screened with Hampton Screen 1 (Hampton Research, Laguna Nigel, CA.) and Wizard Screen 1 (Emerald Biostructures, Bainbridge Island, WA) at room temperature (20°C). 500-

10 μL well volumes were used. A 1:1 ratio of protein-compound mix to the well solution was used in a hanging drop format to complete the screen. After 10 days, but less than 18 days later crystals were observed in Wizard 1/45 (20% PEG 3000, 0.1M NaOAc⁻ pH 4.5). Optimization and seeding efforts around this condition provided crystals that grew in 17-20% PEG 3000, 0.1M Na Acetate pH 4.5. Seeding

15 was done utilizing a cat whisker which was touched to a drop containing microcrystals and stepwise diluted by streaking through one row of the optimization tray. Cross-seeding efforts provided crystals of HEK 293 cell derived protein (38mg/ml in 20mM Hepes pH 7.8, 50mM NaCl, 10% DMSO, 2mM of the inhibitor shown in Figure 1b) from CHO cell derived seed stock. Macroseeding by moving

20 small crystals with a loop from the target drop to a fresh drop containing 17-20% PEG3000, 0.1M Na Acetate pH 4.5 also resulted in crystals that doubled or more in size, usually with a shower of microcrystals also. In most cases the microcrystals adhered to the larger crystal making them unsuitable for use. Crystals were obtained in hanging drop or sitting drop methods by seeding. Crystals obtained from streak

25 seeding attempts were frozen in a cryoprotectant solution based on the mother liquor plus 20% Ethylene Glycol. The crystals were then soaked incrementally through 5% intervals of the cryoprotectant in 3 to 5-minute intervals. Crystals have also been grown in the presence of 10% glycerol or 10% ethylene glycol to facilitate stabilization into cryogenic solutions. In these cases, the crystals were soaked

incrementally through 5% intervals of the cryoprotectant in 3 to 5-minute intervals.

5   **B. X-ray Diffraction Characterization**

Human beta secretase crystals were generally too small for useful data collection using standard x-ray diffraction equipment. Therefore, all data collection was carried out at the Advanced Photon Source (Argonne, IL) at beamline 17-ID. The crystals diffracted to 3.2Å using synchrotron radiation. Crystals were of

10   the space group P32$_2$1 with cell constants a=112 ± 20 Å, b=112 ± 20 Å, c=110 ± 20 Å, $\alpha=\beta=90°$, $\gamma=120°$. The Matthews coefficient for these crystals assuming that there is one molecules in the asymmetric unit is 3.5Å/Da with 65% solvent. The structure determination (see below) revealed the presence of electron density in the active site appropriate for the inhibitor shown in Figure 1(B).

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C. Molecular Replacement

A molecular replacement solution was determined using AMORE (Navaza 1994; Collaborative Computational Project N4, Acta Cryst. D50:760-3 (1994))) by utilizing a previously published model of human beta secretase, 1FKN,

20   (Hong et al. (2000), *Science 290*: 150-153) made available from the Protein Data Bank (www.rcsb.org). Using the 1FKN model, the initial rotation solution gave a single strong peak of 9.7σ with the next strongest peak appearing at 4.0σ. The final determination of the space group (P321, P32$_1$1, or P32$_2$1) was determined experimentally by testing translation searches in each space group. A translation

25   search in the correct space group, P32$_2$1, resulted in a correlation coefficient of 55.1 with an R-factor of 39.9% to 4Å resolution.

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Table 4. Data collection statistics for structure of Human Beta Secretase (data collected at λ 1.0000 Å at APS, 17-ID)

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Resolution Range	R_{sym}
20-6.81	0.048
6.81-5.44	0.095
5.44-4.77	0.112
4.77-4.33	0.113
4.33-4.03	0.181
4.03-3.79	0.224
3.79-3.60	0.248
3.60-3.45	0.288
3.45-3.31	0.321
3.31-3.20	0.324
All reflections	0.098

E. Model Building and Refinement

Further rigid body refinement of the model in CNX (Molecular

5   Simulations, Inc) followed by minimization and group b-factor refinement gave an

R-factor of 35.1% and a Free R-factor of 37.7% to 3.2Å. During each cycle of

refinement a bulk solvent correction was incorporated (J.S. Jiang & A.T. Brunger, J.

Mol. Biol. 243:100-15 (1994)). Progress of the refinement was monitored by a

decrease in both the R-factor and Free R-factor.

10   At this point, inspection of the electron density map within the

active site revealed electron density that was unaccounted for by the protein model

and consistent with the shape of the inhibitor shown in Figure 1B that was present in

the crystallization conditions. Model building was done using the program CHAIN

(J.S. Sack, Journal of Molecular Graphics 6:224-5 (1988)) and LORE (B.C. Finzel,

15   Meth. Enzymol. 277:230-42 (1997)). Modest rebuilding of the model into the 3.2Å

low resolution map afforded the opportunity for further cycles of refinement giving

improvement of the R-factor to 31.6% and a Free R-factor of 35.7% . Finally, the

inhibitor was included in the refinement to give the current R-factor of 29.9% and a

Free R-factor of 34.9%.

20   Inspection of the electron density throughout the molecule indicates

that all three disulfide bonds are intact (Cys155-Cys359,Cys217-Cys382, and

Cys269-Cys 319). In addition, the electron density near Asn92, Ans162, and

Asn293, indicates the presence of glycosylation. Only the glycosylation at Asn111

is disordered enough not to be visible in the electron density map. Residues 158-

25   170 and 311 to 317 were disordered in the electron density and therefore have been

omitted from the model.

Table 5. Refinement Statistics for structure of Human Beta Secretase

	R-factor	Free R-factor	No. of reflections
20-3.2 Å F≥ 2σ	0.2991	0.3483	9883
	Bonds (Å)	Angles(°)	
r.m.s deviation from ideal geometry	0.012	1.7	

	Number of atoms	Average B-factor
Protein	2880	65.24
Waters	41	74.89
Total	2921	65.38

SEQUENCE LISTING FREE TEXT

SEQ ID NO:1   residues for recombinant human beta secretase present in the

X-ray structure

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EMVDNLRGKSGQGYYVEMTVGSPPQTLNILVDTGSSNFAVGAAPHPFLHRYYQR
QLSSTYRDLRKGVYVPYTQGKWEGELGTDLVSIPHGPNVTVRANIAAITESDKFFINGSN
WEGILGLAYAEIARPDDSLEPFFDSLVKQTHVPNLFSLQLCGAGFPLNQSEVLASVGGSM
IIGGIDHSLYTGSLWYTPIRREWYYEVIIVRVEINGQDLKMDCKEYNYDKSIVDSGTTNL
RLPKKVFEAAVKSIKAASSTEKFPDGFWLGEQLVCWQAGTTPWNIFPVISLYLMGEVTNQ
SFRITILPQQYLRPVEDVATSQDDCYKFAISQSSTGTVMGAVIMEGFYVVFDRARKRIGF
AVSACHVHDEFRTAAVEGPFVTLDMEDCGYN
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